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Progress in Clinical Oncolytic Virus-based Therapy for Hepatocellular Carcinoma

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Progress in Clinical Oncolytic Virus-based Therapy for Hepatocellular Carcinoma

Review article

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Abstract

Hepatocellular carcinoma (HCC) carries a dismal prognosis, with advanced disease being resistant to both radiotherapy and conventional cytotoxic drugs, whilst anti-angiogenic drugs are marginally efficacious. Oncolytic viruses (OV) offer the promise of selective cancer therapy through direct and immune-mediated mechanisms. The premise of OV lies in their preferential genomic replication, protein expression and productive infection of malignant cells. Numerous oncolytic viruses are being tested in pre-clinical models of HCC, with good evidence of direct and immune-mediated anti-tumour efficacy. Efforts to enhance the performance of these agents have concentrated on engineering OV cellular specificity, immune evasion, enhancing anti-tumour potency and improving delivery. The lead agent in HCC clinical trials, JX-594, a recombinant Wyeth strain Vaccinia virus has demonstrated evidence for significant benefit and earned orphan drug status. Thus, JX-594 appears to be transcending the barrier between novel laboratory science and credible clinical therapy. Otherwise, relatively few other OV have entered clinical testing, a hurdle that must be overcome if significant progress is to be made in this field.

This review summarises the pre-clinical and clinical experience of OV therapy in the difficult-to-treat area of HCC.

Introduction

HCC is a malignancy of hepatocytes with an annual incidence over 500,000 (Boyle et al., 2008). The majority of HCC cases can be attributed to defined environmental risks; worldwide, the proportion of HCC caused by chronic hepatitis B virus (HBV) infection is approximately 54%, with 31% being attributed to hepatitis C virus (HCV) (Boyle et al., 2008). A safe and effective vaccine exists for HBV with good global coverage ("WHO | Immunization coverage," 2014). In contrast, the highly heterogeneous nature of HCV has hampered attempts at vaccine development. In the UK, HCC caused by alcohol and non-alcohol-related fatty liver disease is on the rise (CRUK, 2013). The majority of patients present with advanced incurable disease and the overall 5 year survival rate is between 5 and 9% (Boyle et al., 2008). Early diagnosis is critical, as patients with localised HCC and a good performance status may be offered potentially curative liver resection or transplantation. The management of patients with advanced HCC is complicated by underlying liver disease and the need to avoid undue toxicity in patients with a poor prognosis. For patients with inoperable predominantly hepatic disease, locoregional therapies offer the potential for disease control, symptomatic relief and improved survival times (Cammà et al., 2002) (Bouza et al., 2009) (Memon et al., 2011). Clinical studies evaluating the use of cytotoxic chemotherapy have typically reported low response rates, with no impact on overall survival (Wrzesinski et al., 2011). The current standard of care for patients with metastatic HCC is sorafenib, an oral multi-kinase inhibitor with anti-proliferative and anti-angiogenic

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properties. It confers a mean survival advantage of 3 months in comparison to best supportive care (BSC), but rarely induces radiological responses, and is associated with significant toxicity (Cheng et al., 2009) (Llovet et al., 2008).

Immunotherapies are a promising class of drugs that include OV, therapeutically useful viruses that preferentially replicate in, and kill cancerous cells. Growing evidence suggests that effective oncolytic virotherapy is unlikely to be achieved merely by direct infection and cell lysis, but rather through efficient stimulation of an anti-cancer immune-response as reviewed by Melcher et al., 2011. To date, hundreds of patients with HCC have been treated using OV in phase 1 and 2 clinical trials. The emerging data is encouraging both in terms of the relatively favourable side-effect profiles and early signs of efficacy. The current lead agent, JX-594 also known as Pexa-Vec (pexastimogene devacirepvec) was granted orphan drug status in HCC by the U.S. Food and Drug Administration in 2013 and by the European Medicines Agency in 2009 (France, 2013). Orphan drug designation (ODD) is approved for drugs that seek to treat rare diseases for which there may be few adequate therapies, and comes with incentives that include marketing exclusivity, grant funding for clinical trials and tax credits for clinical research expenses. Whilst these incentives assert the dominance of JX-594 in the field, they have not perturbed the translational development of other OV for HCC therapy. In addition to JX-594, three other OV have been or are currently being tested in HCC-directed clinical trials, including two based on type 5 adenoviruses, dl1520 (ONYX-015) (Habib et al., 2002) and H101 (Oncorine) [NCT01869088] as well as a vesicular stomatitis virus (VSV) encoding the human interferon (IFN)-β gene (VSV-hIFN-β) [NCT01628640].

This review summarises the pre-clinical and clinical progress of oncolytic virotherapy in HCC, focussing on the molecular methods employed to improve virus targeting to malignant hepatocytes, the use of virus-encoded therapeutic genes, and methods to improve viral survival. We also summarise the completed and ongoing clinical trials, routes of clinical viral delivery, and published clinical safety and efficacy data.

Engineering Oncolytic Viruses for HCC Therapy

Although the first wave of OV clinical trials took place in the 1950s and 1960s, it was not until the 1990s that engineered OV blossomed alongside advances in DNA manipulation and molecular biology techniques.

Targeting Malignant Hepatocytes

The specificity of any given drug determines its side-effect profile and greatly influences its efficacy. JX-594, dl1520, H101 and VSV-hIFN-β have all been engineered for pan-cancer specificity, targeting hallmark cancer characteristics, such as TP53 deletion and upregulated thymidine kinase (TK)

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expression. Other engineered pan-cancer specific OV have also shown efficacy in pre-clinical HCC models, including those whose genome expression is driven by survivin, an inhibitor of apoptosis protein family that is overexpressed in the majority of HCC cases (survivin promoter-regulated oncolytic adenovirus vector carrying TP53 gene, AdSurp-P53) and human telomerase reverse transcriptase (hTERT), expressed in up to 90% of HCCs, but only some 20% of non-malignant liver cells (hTERT promoter-regulated replicative adenovirus, SG300) (Kannangai et al., 2005) (He et al., 2012) (Nagao et al., 1999) (Liu et al., 2011). More recently, OV that preferentially target tumour initiating cells have been engineered, including oncolytic measles virus retargeted to CD133 positive cells (Bach et al., 2013). In the liver, CD133 expression is limited to cancerous tissue, and is associated with colony formation and high proliferative capacity (Kohga et al., 2010) (Zhu et al., 2010).

In contrast to pan-cancer specific OV, numerous pre-clinical OV have been engineered to specifically target HCC (table 1). Commonly, HCC-specific viral promoters are inserted into the viral genomes that restrict the transcription of viral genes to HCC cells and hence limit the destruction of healthy cells (Ohguchi et al., 1998) (Foka et al., 2010). Viral gene expression in these systems can be further increased by the insertion of an insulator element upstream of the HCC specific promoter, to shield from viral silencers, while retaining specific gene expression in hepatoma cells (Ye et al., 2003).

A further approach to specifically target malignant hepatocytes is to exploit the differential expression of micro-RNA (miRNA) transcripts; recently, a 30 miRNA signature consisting of 10 down-regulated and 20 up-regulated miRNAs was established for distinguishing HCC from non-cancerous liver tissues (Wei et al., 2013). Complementary sequences to miRNA transcripts that are specifically down-regulated in HCC e.g. mir-122 and mir-199 have been inserted into the 3'untranslated regions of OV including oncolytic type 5 adenovirus and HSV (Cawood et al., 2009) (Fu et al., 2012) (Khalid Elamin Elhag, 2012). The resulting selective viral RNA degradation in normal hepatocytes led to decreased hepatotoxicity, whilst retaining anti-HCC potency in animal models.

These methods are not without their problems as shown in table 1, and the protein or miRNA-binding site to be engineered into the OV genome must be chosen wisely.

Enhancing Anti-Cancer Efficacy

Numerous therapeutic anti-cancer genes have been engineered into oncolytic viruses in a bid to enhance efficacy. In particular, replication competent adenovirus vectors have been extensively modified and tested in pre-clinical models of HCC as illustrated in Fig. 1. The engineered therapeutic genes fall under one of two broad categories: those that modify the tumour microenvironment including stimulation of anti-HCC immune responses, and those acting directly on HCC cells to induce apoptosis and reduce cell growth and survival. In addition to the examples shown in figure 1,

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both oncolytic measles and Newcastle disease viruses have been engineered to express enzymes that convert the prodrug 5-fluorocytosine into the active chemotherapeutic 5-fluorouracil, enabling OV-mediated targeted chemotherapy, significantly enhancing OV efficacy (Lv et al., 2013) (Lampe et al., 2013). The majority of approaches to arming OV can be equally applied in the treatment of any solid malignancy. Exceptions include recombinant human erythropoietin (rhEPO), which was recently engineered into an oncolytic Lister strain Vaccinia virus (GLV-1h210) and tested in lung cancer xenografts (Nguyen et al., 2013). rhEPO is essential for erythropoiesis and significantly improves quality of life in anaemic cancer patients, but is associated with angiogenesis, limiting its use in highly vascularised tumours, such as HCC (Yasuda et al., 2003) (Crawford et al., 2002).

A large body of evidence gathered both from pre-clinical and clinical studies in various cancer types points to the potential of OV to stimulate both innate and adaptive anti-cancer immune responses (Melcher et al., 2011) (Prestwich et al., 2008a) (Prestwich et al., 2009). This could also be important for OV therapy in HCC. However, the liver is an immunologically privileged organ, skewed towards an environment of immunological tolerance rather than immunity, as evidenced, for example, by reports of the acceptance of liver allografts across major histocompatibility barriers without immunosuppressive therapy (Seyfert-Margolis & Turka, 2008). This immunosuppressive microenvironment is further compounded in HCCs that frequently harbour enriched regulatory T-cells, elevated immunosuppressive cytokines such as transforming growth factor (TGF)-β and interleukin (IL)-10, and decreased immunostimulatory cytokines such as IL-2 and IFN-γ (Shirabe et al., 2010). In addition, frequently impaired functional activities of NK cells in HCC are associated with poor prognosis (Wada et al., 1998) (Wu et al., 2013).

Encouragingly, HCCs with a more favourable immune microenvironment, including NK cell accumulation are associated with improved survival, and pre-clinical evidence exists for the infiltration of HCC by NK cells following OV therapy, whilst the depletion of NK cells inhibits OV-mediated anti-HCC effects (Chew et al., 2009) (Gentschev et al., 2011) (Tsuchiyama et al., 2007) (Kwon et al., 2001). Several cytokines that have the potential to stimulate anti-cancer NK cell responses have been engineered into OV; IL-12 induces the proliferation and activation of NK cells, in addition to the differentiation of naïve CD4+ T-cells into Th1 cells (Hamza et al., 2010). Similarly, chemokine (C-C motif) ligand 5 (CCL5) also drives the cytolytic activity of NK cells, and induces NK cell proliferation through T-cell mediated IL-2 secretion (Taub et al., 1995) (Maghazachi et al., 1996). Whilst IL-12 and CCL5 have shown promising anti-HCC effects in pre-clinical models, others including IFN-β, a powerful stimulator of NK cell activation, are currently being tested in patients with advanced HCC (NCT01628640).

Key to priming successful T-cell anti-HCC responses are antigen presenting cells (APCs), of which dendritic cells (DCs) are of utmost importance. It is known that DCs from HCC patients have

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significantly lower capacity to stimulate T-cells than DCs from patients with liver cirrhosis or normal controls (Ninomiya et al., 1999). Furthermore in chronic viral hepatitis, there are decreased DC liver populations and impairment in DC capacity to prime naïve T-cells, contributing to the inadequate adaptive immune responses observed (Kanto et al., 2004) (Averill et al., 2007). OV are capable of driving successful T-cell anti-cancer therapy as shown in melanoma models utilising oncolytic wild-type reovirus and VSV-GFP (Prestwich et al., 2008b) (Wongthida et al., 2011). In HCC pre-clinical models, the oncolytic Vaccinia virus GLV-1h68, encoding several biomarker genes only (see table 3), has been shown to promote the intense infiltration of DCs into both HBV positive and hepatitis virus negative xenografts, whilst VSV-GFP promoted the infiltration of DCs into HCC tumours in an orthotopic immunocompetent animal model (Gentschev et al., 2011) (Shinozaki et al., 2005). Although not a prerequisite for successful T-cell therapy, the OV-mediated expression of engineered immunostimulatory genes has the potential to greatly improve efficacy. Several approaches to enhance DC maturation/activation have been tested in pre-clinical HCC models, and include arming viruses with granulocyte macrophage colony-stimulating factor (GM-CSF) or CpG-rich sequences, the latter of which has been shown to increase IFN-γ and DC activation in draining lymph nodes, resulting in improved therapy against hepatoma lung metastases in comparison to the wild-type virus (Raykov et al., 2008). Other groups have shown enhanced DC and CD4+ T-cell tumour infiltration using Vaccinia viruses encoding CCL5 or a secretory bispecific T-cell engager consisting of two single- chain variable fragments specific for CD3 and the tumour cell surface antigen EphA2 (Li et al., 2011) (Yu et al., 2014).

Immune cell recruitment and activation also plays a prominent role in the OV-induced disruption of tumour-associated vasculature. Indeed, inflammation-mediated disruption of vasculature is a well-documented phenomenon (Bryant et al., 2005) (Lee & Slutsky, 2010). VSV infection of subcutaneous tumours resulted in transcriptional activation of the neutrophil chemoattractants CXCL1 and CXCL5, inducing tumour infiltration by neutrophils, vascular shutdown and the apoptosis of uninfected tumour cells (Breitbach et al., 2007). The depletion of neutrophils prior to VSV infection abrogated these effects. In addition to the role played by OV-induced inflammation, JX-594 has been shown to directly infect and kill tumour-associated vascular endothelial cells in mice following intravenous delivery (Breitbach et al., 2013). These findings have been confirmed in human HCC trials, demonstrating disruption of tumour perfusion following JX-594 therapy (Liu et al., 2008) (Heo et al., 2011).

The effects of OV on the wider HCC microenvironment is complex and has recently been reviewed elsewhere (Altomonte & Ebert, 2014).

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Enhancing viral delivery and viral survival

Perhaps the biggest challenge to successful oncolytic virotherapy in HCC is the ability to infect sufficient numbers of malignant hepatocytes with a sufficiently high multiplicity of infection and to maintain viral propagation. It is well established that the immune response to OV is likely to play a dual role; simultaneously clearing the virus and hence limiting efficacy, whilst at the same time becoming more activated and primed to attack malignant cells (Melcher et al., 2011). It is known that adenovirus is rapidly removed following IV delivery by Kupffer cells, liver resident macrophages, and the same may be true of other viruses (Tao et al., 2001). A number of novel methods have been employed to enhance systemic viral delivery to the desired target including Kupffer cell depletion using replication-defective adenovirus, prior to replication-competent adenovirus therapy, and warfarinisation to block coagulation factor and complement dependent binding of adenovirus to hepatocytes (Shashkova et al., 2008). Combined Kupffer cell depletion and warfarinisation resulted in decreased hepatotoxicity and increased anti-tumour potency, albeit in subcutaneous xenografts (Shashkova et al., 2008). A different approach that has been tested in pre-clinical models of HCC is to engineer OV to evade immune inactivation (table 2). These engineered OV are yet to be tested in clinical trials and it remains to be seen whether they paradoxically result in reduced immune-mediated anti-cancer efficacy.

Engineered OV Tested in HCC-Directed Clinical Trials

In addition to the plethora of engineered oncolytic adenoviruses, a large number of wild-type and recombinant OV have been investigated in pre-clinical models of HCC, but are yet to enter HCC-directed clinical trials (table 3). Some of these viruses are clinical-grade agents that have been employed in other anti-cancer clinical trials, and are hence the more likely to proceed to HCC-directed trials.

The following sections describe the OV that have entered HCC-directed clinical trials to date:

JX-594

JX-594 was first filed for patent in 2005 by Jennerex Biotherapeutics ULC; a company that entered into a commercialization and development agreement for JX-594 with Transgene in 2010 and was later acquired by SillaJen Inc. in 2013 (Kirn, 2006) (Transgene, 2010) (Transgene, 2013a). The Wyeth strain of Vaccinia virus, that forms the backbone of JX-594 was derived from the poorly pathogenic New York City Board of Health strain. The Wyeth strain was extensively employed as a smallpox vaccine in the U.S. until routine vaccination was rescinded in 1971 (ODC, 1971). JX-594 has been genetically modified by the homologous recombination of a pSC65 plasmid with the Vaccinia virus TK gene. The plasmid sequence contains the human GM-CSF gene under the control of a synthetic

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early-late promoter and the LacZ reporter gene (Mastrangelo et al., 1998). GM-CSF induces direct anti-tumour effects and importantly influences the immune system through the stimulation, recruitment and maturation of dendritic cells (Urdinguio et al., 2013) (Mach et al., 2000).

The expression of TK, an enzyme of the DNA precursor pathway, is strictly regulated during the normal cellular cycle, but is much higher and permanently expressed in malignant growing cells (Hengstschläger et al., 1998). Being TK deleted, JX-594 cancer-selectivity was believed to be dependent on elevated cellular TK levels in cancers. However, recent work has shown JX-594 cancer specificity to be multi-mechanistic, with replication being dependent on epidermal growth factor receptor/Ras /mitogen-activated protein kinase pathway signalling, cancer cell resistance to type-I interferons, as well as cellular TK levels (Parato et al., 2012).

VSV-hIFN-β

VSV is a negative-strand RNA virus that is non-pathogenic to humans. Effective immune defence to VSV is dependent on the host interferon response, with mice harbouring defective interferon systems succumbing to normally harmless VSV exposure (Durbin et al., 1996). Insertion of genes between the viral glycoprotein and polymerase genes does not affect the fitness of the resultant recombinant virus (Fernandez et al., 2002). Generation of VSV-hIFN-β is achieved by insertion of the human (h)IFN-β gene into the same position of the full-length viral antigenomic cDNA, pVSV-XN2, using unique restriction enzyme sites (Obuchi et al., 2003). The expression of hIFN-β renders successful virus propagation dependent on defective cellular interferon response pathways, as found in many cancers (Barber, 2004). In addition, expression of hIFN-β is envisaged to activate NK and T-cells and facilitate the maturation of DCs for immune-mediated anti-tumour therapy, as well as directly inhibiting malignant cell proliferation (Odaka et al., 2001) (Ferrantini & Belardelli, 2000) (Kadowaki et al., 2000). VSV-hIFN-β is patented and being developed by the Mayo Foundation for Medical Education and Research (Federspiel et al., 2010).

dl1520 (ONYX-015) and H101 (Oncorine)

The adenovirus type 5 early regions 1A (E1A) and 1B (E1B) can be exploited to engineer cancer specificity; the protein products of E1A induce cellular DNA synthesis and transformation, but trigger apoptosis mediated by the mammalian tumour cell suppressor protein p53, with a resultant reduction in the yield of progeny (Bayley & Mymryk, 1994) (Debbas & White, 1993). The 55-kDa E1B protein binds to the p53 protein and blocks p53-mediated transcriptional activation, hence limiting p53-dependent cell cycle arrest and apoptosis (Sarnow et al., 1982) (Yew & Berk, 1992). Early gene-therapy adenoviral type 5 vectors were modified to disable productive infection by the deletion of both E1A and E1B. These replication deficient adenovirus vectors were extensively used in cancer gene therapy trials, however evidence for efficacy was restricted due to self-limiting transgene

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expression, poor target cell transduction and lack of tumour cell targeting (Vile et al., 2000). On the other hand, disabling the E1B region alone theoretically leads to selective replication in p53-deficient cells. One of the first such replication-selective type 5 adenoviruses, dl1520 has an 827-base pair deletion in the E1B region and a point mutation at codon 2022 that generates a stop codon preventing expression of a truncated protein from the deleted gene (Barker & Berk, 1987).

Initial data suggested that dl1520 does indeed selectively replicate in TP53-deficient cells (Bischoff et al., 1996). However, it is now accepted that TP53 status is in fact a poor predictor of the sensitivity of tumour cells to dl1520 with tumour specificity being determined by other factors such as the inhibition of viral RNA export in non-malignant cells (Edwards et al., 2002) (O'Shea et al., 2004). An incomplete understanding of the mechanisms of OV cancer specificity can hamper clinical progress, as exemplified by a trial testing dl1520 in hepatobiliary cancers, where patients with HBV infections were in hindsight unnecessarily excluded due to theoretical risks that HBV protein X can inactivate p53 protein in non-malignant hepatocytes, rendering them susceptible to dl1520 productive infection (Makower et al., 2003).

dl1520 was clinically developed by Onyx Pharmaceuticals under the name ONYX-015 until 2003 when a promising phase 3 trial in head and neck cancer was suspended. Exclusive rights to ONYX-015 were sold to Shanghai Sunway Biotech in 2005 (Investis, 2005). In the years preceding this acquisition, Shanghai Sunway Biotech was simultaneously developing H101 (Oncorine), a recombinant human adenovirus type 5 similar to ONYX-015. In November 2005, the Chinese State Food and Drug Administration approved H101 for advanced nasopharyngeal carcinoma in combination with chemotherapy (Medscape, 2005). Like dl1520, H101 is E1B gene deleted, but unlike dl1520, H101 has an additional partial E3 78.3-85.8μm gene segment deletion (Lu et al., 2004). E3 gene products prevent T-cell and NK cell recognition of infected cells by preventing transport of MHC class I to the plasma membrane and by sequestration of MHC class I-related molecules A and B respectively (Burgert & Kvist, 1985) (McSharry et al., 2008). The partial E3 gene deletion in H101 is thought to enhance its safety profile, although this may be at the cost of decreased anti-cancer potency (Suzuki et al., 2002).

Clinical Experience of Oncolytic Virus-Based Therapy in HCC

To date only 4 HCC-directed clinical trials using two different OV, JX-594 and dl1520 have been undertaken and completed follow-up (table 4). Early phase trials that include a mixed population of patients with digestive tract tumours, have typically recruited very small numbers of patients with HCC, making it difficult to adequately characterise the performance of these agents (Park et al., 2008)

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(Habib et al., 2001). It is also noteworthy that patients with significant chronic infections including HIV, HBV and HCV infection are frequently excluded from trials of OV that include multiple disease sites, primarily due to the perceived risk of increased adverse events (Pecora, 2002) (Vidal et al., 2008). Encouragingly, at least 3 other OV trials exclusively for HCC are either underway or nearing completion (table 5).

Route of Delivery

The safety and efficacy of OV therapy is dependent not only on viral specifics, but also on numerous clinical considerations, including the administered dose of virus, the rate of infusion, the anatomical distribution of disease and the route of delivery.

Intratumoural Injection

Numerous intratumoural (ITu) therapies have been trialled in liver tumours, and it is a popular OV delivery method in HCC (see tables 4 and 5) (Venook, 2000). The advantages of the ITu route are the delivery of a high concentration of drug to the target, whilst minimising off-target side-effects, an important consideration in HCC where the background liver is frequently cirrhotic with reduced functional capacity. However, direct ITu injection carries significant risks of bleeding, infection, peritoneal tumour seeding as well as technical challenges. It is frequently impossible to inject all HCC foci, but this is not necessarily a limitation of the technique; Park et al reported that ITu injection of JX-594 led to the initial release of virus into the bloodstream, that was rapidly cleared (Park et al., 2008). This was then followed by the re-emergence of circulating JX-594 days to weeks later, consistent with productive infection. In keeping with these observations, replicating JX-594 infection was found in a non-injected HCC focus metastatic to the neck following ITu liver injection (Park et al., 2008).

Intravenous Injection

The IV delivery of OV avoids the local injection-site side-effects associated with invasive ITu therapy. IV injection is also more likely to be acceptable to both patients and their physicians when administered at regular intervals as part of a scheduled course of treatment. Intravenous administration of JX-594 has been shown to result in viral delivery to tumours, with the key determinant of tumour infection being the administered dose (Breitbach et al., 2011). Of the patients treated with doses $\geq 1.5 \times 10^7$ PFU kg^{-1} and subsequently biopsied, 87% showed JX-594 positivity in tumour by IHC or qPCR, whereas those treated with lower doses were negative. All patients on this trial had a history of vaccination with live Vaccinia virus as children, and delivery was demonstrated in a patient despite the presence of neutralizing antibodies at baseline. This finding lends support for the need to establish the maximum tolerated dose in trials of oncolytic virotherapy and to use the maximum tolerated dose in subsequent phase 2 and 3 trials. The IV route is further supported by a translational trial where oncolytic reovirus was recovered post-surgery from colorectal cancer liver

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metastases following IV delivery, and shown to be capable of plaque formation ex-vivo (Adair et al., 2012). In the same trial, no replicating reovirus was recovered from normal liver samples, but faint staining for reovirus sigma 3 protein was seen by IHC, supporting the notion of preferential productive infection in cancerous tissue.

Several trials have employed an initial IV injection of OV followed by ITu injections. The theory behind this approach is that initial IV injection will prime an immune response that is then amplified at the target site upon further ITu injections.

Hepatic Artery Injection (HAI)

HAI using cytotoxic agents is in routine clinical practice for patients with HCC and warrants further investigation in oncolytic virotherapy. This is commonly employed in the form of transarterial chemoembolization (TACE), either as a palliative technique per se, or as a 'bridging' modality before liver transplantation (Jelic & Sotiropoulos, 2010). The TACE principle employs HAI of cytotoxic drug combinations followed by lipiodol or degradable microsphere injection for vessel occlusion, resulting in tumour cell ischaemia and necrosis.

It is debateable whether HAI enhances viral delivery to localised targets over the simpler method of ITu injection. HAI also does not prevent systemic side-effects as was significantly highlighted by the well-publicised death of the teenager Jesse Gelsinger secondary to systemic inflammatory response syndrome (SIRS) induced by the hepatic artery injection of $3.8x10^{13}$ virus particles of replication incompetent adenovirus type 5 (E1 and E4 deleted) encoding ornithine transcarbamylase cDNA (Raper et al., 2003). The strength of HAI lies in the opportunity to improve on existing locoregional therapies in combination with TACE, and encouragingly, a phase 3 trial of H101 in combination with TACE in patients with HCC is currently recruiting (table 5). Clearly, further trials testing OV by HAI are warranted and it remains to be seen which route of delivery is preferable in terms of safety, efficacy and patient acceptability.

Clinical Safety Data

As can be seen from table 4, both ITu and IV injections of JX-594 have been tested in patients with HCC. The most common adverse events are an influenza-like illness comprising headache, nausea, vomiting and fatigue (Park et al., 2008) (Breitbach et al., 2011) (Heo et al., 2013a). A mild fever occurs in all patients and is dose-related (Heo et al., 2013a). A maximum tolerated dose was reached at 10^9 PFU due to grade III hyperbilirubinaemia subsequent to transient tumour swelling inducing biliary obstruction (Park et al., 2008). Peri-tumoural oedema, induced by acute inflammation has been commonly reported in trials using OV and in fact response after initial tumour flare is a class effect of immune therapies in general (Pecora, 2002) (Senzer et al., 2009) (Wolchok et al., 2009). The absence

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of substantial changes in AST and ALT suggest that direct destruction of healthy hepatocytes following JX-594 injection is mild (Park et al., 2008).

Habib et al reported safety data from 10 patients with HCC treated with dl1520. Following a dose-escalation study in patients with either primary or secondary liver tumours in which no maximum tolerated dose was reached, a further small HCC-directed trial was undertaken in Egypt (Habib et al., 2001). In the latter study 10 patients were randomised in a 1:1 ratio to receive either a single IV dose of $3x10^{11}$ PFU of dl1520 followed by 5 ITu doses, or standard of care therapy with 95% ethanol by ITu injection (Habib et al., 2002). Of the five patients treated with dl1520, three suffered from CTCAE grade I-II fever and rigors, and 2 patients suffered from transient hypotension at the time of the infusions. Very minor changes in AST and ALT were observed for patients treated with dl1520, in comparison to the much higher levels of serum transaminases observed following ethanol treatment (Habib et al., 2002).

Assessing Efficacy in OV Therapy for HCC

For the approval of new anti-cancer drugs, the FDA accepts improved survival, as well as surrogate markers that predict clinical benefit. The Response Evaluation Criteria in Solid Tumours (RECIST) use single linear summation of target lesions to define response to therapy (Therasse et al., 2000). However, the clinical benefit provided by anti-cancer therapy in HCC correlates poorly with conventional methods of response assessment (Llovet et al., 2008) (Forner et al., 2009). In 2008, the American Association for the Study of Liver Diseases (AASLD) developed a set of guidelines, termed the modified RECIST or mRECIST criteria aimed at providing a common framework for the design of clinical trials in HCC (Lencioni & Llovet, 2010). These guidelines consider estimation of the reduction in viable tumour area using contrast-enhanced radiologic imaging to be the optimal method to assess treatment response in HCC. Nonetheless, both RECIST and mRECIST criteria must be employed with caution in trials using immunotherapies; in particular, OV may cause transitory tumour-flare secondary to inflammatory cytokine release, leading to tumour enlargement and increased contrast enhancement, prior to tumour necrosis and shrinking (Senzer et al., 2009). Delaying radiologic assessment following OV therapy could potentially avoid this issue (Hales et al., 2010).

Clinical Evidence of Anti-Tumour Efficacy

In a recent pivotal study, 30 patients with advanced HCC were randomised to low (10^8 PFU) or high dose (10^9 PFU) intratumoural JX-594 administered every 2 weeks (Heo et al., 2013a). The majority of patients in both groups had previously received locoregional therapy, but more patients in the high dose group had previously failed sorafenib therapy, a poor prognostic factor. Median overall survival was 14.1 months for the high dose arm and 6.7 months for the low dose arm. Despite the relatively

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small sample size, a statistically significant survival benefit (P=0.020) was demonstrated because of the large effect size. Both doses were associated with mRECIST responses, decreased tumour perfusion and decreased tumour contrast enhancement. This is the first study to show a statistically significant benefit derived from OV therapy in patients with HCC.

JX-594 has been tested as second line therapy in two phase 2 HCC trials (see table 4). In the larger of these studies (TRAVERSE), patients who had previously failed sorafenib therapy were treated with JX-594 and BSC or BSC alone (Transgene, 2013b). Sadly, the primary endpoint of improved overall survival was negative. The failure of JX-594 in the TRAVERSE trial following promising randomised dose-finding trial data remains to be fully explained. Patients recruited to the TRAVERSE trial were more likely to have sorafenib-resistant cancers. Acquired cellular resistance mechanisms to sorafenib following long-term exposure include compensatory crosstalk between PI3K/Akt and MAPK pathways, upregulation of the JAK-STAT pathway and enhanced epithelial-mesenchymal transition (Zhai & Sun, 2013). These changes could theoretically affect OV infection and anti-cancer efficacy, although recently, the modified Lister strain Vaccinia virus, GLV-1h68, was shown to effectively infect and kill sorafenib-resistant HCC cell lines (Ady et al., 2014). Alternatively, the failure of JX-594 in the TRAVERSE trial could be attributed to more advanced disease in the second line setting; fitter patients carrying a smaller HCC disease burden are most likely to respond to OV therapy, as has been the experience with other immunotherapies (Coppin et al., 2005). Furthermore, the relatively small number of patients included in phase 2 trials presents a challenge when seeking outcomes of study drug superiority over standard care. Nonetheless, Transgene recently announced a shift in strategy, moving JX-594 trials away from the second-line setting in HCC. Instead, a phase 3 trial which is expected to enrol approximately 600 patients and is anticipated to begin recruitment in 2015, will be testing whether first line IT JX-594 (weeks 0, 2 and 4) followed by sorafenib (week 6 onwards) improves overall survival in comparison to sorafenib alone (Transgene, 2014).

In contrast, no meaningful efficacy data can be derived from the dl1520 trial by Habib et al; one patient who received dl1520 experienced a partial response with reduction in tumour volume from 306 to 22.5 cm^3 associated with a concomitant decrease in AFP level from 7604 to 300 ng mL^{-1} (Habib et al., 2002). The remaining four patients demonstrated progressive disease with an increase in both tumour volume and AFP levels. Larger randomised trials are needed to determine whether recombinant type 5 adenoviruses are efficacious in HCC.

Clinical Evidence of Anti-cancer Immune Stimulation

Anti-cancer immune stimulation could be at least partially responsible for the reported decreases in the size and contrast enhancement of non-injected tumours following intratumoural JX-594 injection elsewhere (Park et al., 2008) (Heo et al., 2013a). However, little ex-vivo evidence has been gathered to date from clinical trials for anti-HCC immune responses. In their randomised dose-comparison

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phase 2 trial, Heo et al demonstrated HCC immune infiltration following JX-594 injection by both radiographic peripheral tumour enhancement and histologically confirmed diffuse lymphocyte infiltration from biopsied tumours (Heo et al., 2013a). In the same trial, Heo et al assessed antibody-mediated complement-dependent cytotoxicity (CDC) by the addition of serum from JX-594 treated patients to HCC cell lines, resulting in cytotoxicity from 11 of the 16 subjects tested (Heo et al., 2013a). Indeed, CDC could be of vital importance in OV therapy as evidenced by a recent JX-594 study in patients with a variety of cancer types, where patients with the longest survival duration had the highest CDC activity (Kim et al., 2013). Evidence was also gathered for antibody development and T-cell immunity against JX-594 encoded proteins including β-galactosidase, an observation of likely importance in the elimination of virus-infected tumour cells (Heo et al., 2013a). Whilst encouraging, these results do not constitute an adaptive anti-HCC immune response. At least 6 different HCC-specific tumour associated antigens (TAA) that are targeted by T-cells have been identified and future OV trials should assess whether specific T-cell responses against these antigens are induced (Breous & Thimme, 2011).

Other evidence for immune stimulation is similarly encouraging, though sparse; both elevated TNF-α and IFN-γ have been observed in the serum of HCC patients treated with JX-594 (Liu et al., 2008) (Park et al., 2008). These are likely to contribute to DC maturation, cancer growth inhibition and apoptosis. Of interest, the presence of type I interferons, powerful stimulators of NK cell activity and DC maturation, has not been reported in JX-594-treated patients, perhaps due to efficient Vaccinia virus-mediated inhibition of the interferon system (Perdiguero & Esteban, 2009). In contrast, other viruses e.g. measles, reovirus and VSV, are known to efficiently induce type I interferons, wetting the appetite for HCC clinical trials in HCC with thorough translational read-outs using such agents (Steele et al., 2011) (Diaz et al., 2007) (Donnelly et al., 2013). One potential concern is that co-infection of HCV infected hepatocytes with OV will not lead to robust interferon induction due to the interferon evasion mechanisms employed by HCV. For example, HCV NS3/NS4a protease disrupts pattern recognition receptor signalling by cleaving the RIG-I and TLR3 downstream adaptors, MAVS and TRIF respectively (Foy et al., 2005) (Li et al., 2005b) (Ferreon et al., 2005). NS3/NS4A also perturbs RIG-I downstream signalling through disruption of virus-induced NF-κB binding to the DNA PRDII element, hence limiting IFN-β gene expression (Foy et al., 2005) (Li et al., 2005c). Realistically however, the scenario of reovirus co-infection with HCV is unlikely to be a major factor in HCC patients, as the majority of patients only have detectable HCV proteins or genomes in a minority of clustered hepatocytes (Stiffler et al., 2009) A further concern is that HCV and HBV could supress OV-mediated adaptive anti-tumour immune responses, however, no clinical evidence for this yet exists, and future HCC-directed trials cannot afford to exclude the majority of HCC patients, with a viral aetiology.

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Future Perspectives

The clinical progress of JX-594 in HCC therapy provides much optimism in the field. This agent appears to be transcending the barrier between novel laboratory science and credible clinical therapy. From this clinical progress have come clues to support existing laboratory research into the mechanisms of OV-mediated anti-HCC efficacy including the direct, immune and anti-vascular effects. However, much remains to be discovered in terms of the differential response to OV therapy in subsets of patients, the optimal route of delivery and combinations with other anti-cancer therapies. Furthermore, biomarkers predictive of treatment response are greatly needed, as are continued efforts to establish early diagnoses of cirrhosis and HCC using technologies such as the non-invasive enhanced liver fibrosis test (Lichtinghagen et al., 2013).

The combination of OV with sorafenib warrants particular mention. These drug combinations have non-overlapping toxicities, and potentially synergistic mechanisms of action, hence forming the focus of past and future trials. For JX-594, the sequence of this combination is of paramount importance; upfront JX-594 therapy is thought to induce acute vascular disruption, sensitising tumours to the anti-angiogenic effects of subsequent sorafenib treatment. In murine tumour models, sequential JX-594 followed by sorafenib therapy was superior to either simultaneous therapy or sorafenib followed by JX-594 (Heo et al., 2011). In vitro, sorafenib, a multi-kinase inhibitor, perturbs JX-594 productive infection of HCC cell lines, a result that can be predicted as sorafenib inhibits a wide range of cellular kinases in addition to its principal targets, whereas Vaccinia viruses are known to encode kinases, including B1R and TK that are essential for productive infection (Rempel & Traktman, 1992) (Parato et al., 2012) (Kitagawa et al., 2013). The very fact that the cancer specificity of JX-594 is partially dependent on elevated TK levels in malignant cells highlights the reliance of this OV on functional viral and cellular kinases. Hence, sequential scheduling works best for this OV, as was employed in the second line trial using JX-594 followed by sorafenib therapy, and a similar schedule is planned for the first line phase 3 trial (Heo et al., 2011) (Transgene, 2014).

The combination of other OV that are less reliant on cellular kinase functions with sorafenib should form the focus of future studies. The precise scheduling should be determined by preclinical studies in immunocompetent animal models. Kottke et al., showed that tumours treated in vivo with VEGF inhibitors became highly susceptible to systemic treatment with reovirus, but only if the drugs were withdrawn 24–48 hours before virus delivery. The authors concluded that the rebound of VEGF signalling upon drug withdrawal conditions tumour-associated endothelium for productive infection of reovirus (Kottke et al., 2010).

The complex immunomodulatory effects of sorafenib are also likely to be critical determinants of success. One report cited that sorafenib significantly reduced the number of NK cells and inhibited

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their reactivity against tumour targets in animal models, whilst a contradictory report stated that sorafenib enhances IL-12 secretion from human liver-derived macrophages, hence activating NK cells (Sprinzl et al., 2013) (Zhang et al., 2013). The efficacy of OV in combination with sorafenib will therefore be partially dependent on the stimulation or suppression of immune responses. Sorafenib could theoretically enhance OV therapy through a number of mechanisms including the synergistic activation of NK cells, and inhibition of the OV-directed humoral response, thus enhancing IV delivery, as has been the experience with chemotherapy (Lolkema et al., 2011). Alternatively, sorafenib-induced immunosuppression could limit the immune-mediated efficacy of OV, whilst immune stimulation could limit virus propagation, both resulting in reduced efficacy. Orthotopic immunocompetent animal models could begin to answer these questions, but the lack of concordance between animal models and human research highlights the need to pursue early phase clinical trials using sorafenib-OV combinations.

In addition to sorafenib, numerous successful preclinical studies have been conducted, using OV in combination with cytotoxic agents, radiotherapy and targeted biotherapies including other pre-clinical OV (Mao et al., 2009) (Zheng et al., 2009) (Chung et al., 2002). More recently, antibodies targeting the immune checkpoint molecules, CTLA-4 and PD-1/PD-L1 have been tested in early-phase HCC-directed clinical trials (Sangro et al., 2013a) (Sangro et al., 2013b). CTLA-4 is expressed on T-cells and inhibits T-cell activation, whilst PD-1/PD-L1 interactions limit the activation of NK, B- and T-cells (Pardoll, 2012). Combinations of OV with immune checkpoint inhibitors are being explored in solid and haematological malignancies and should also be tested in HCC, with the premise that OV-mediated tumour vaccination, followed by immune activation through checkpoint inhibition may prove highly beneficial (Engeland et al., 2014) (Minev et al., 2014). As with all combination regimens, overlapping side effects are of concern, especially severe immune-related toxicity. HCC therapy provides the opportunity to limit systemic side-effects by HAI, a delivery method that is likely to become increasingly important in future trials.

Taking these combinations one step further, future studies should assess the efficacy of OV carrying cDNA libraries, in combination with checkpoint inhibitors. Effective cancer immunotherapy requires the release of TAA in the context of potent immune activation. Kottke et al., showed that a cDNA library of normal tissue, expressed from oncolytic VSV, acting as an immune adjuvant, cured established tumours of the same histological type from which the cDNA library was derived (Kottke et al., 2011). In HCC therapy, such broad antigenic stimulation can potentially lead to the attack of healthy hepatocytes. This problem can be avoided by engineering OV to express specific TAA including AFP, EpCAM and SSX-2. Clues to indicate the likely efficacy of the latter approach can be found in patients with HCC who have a better prognosis, associated with the expression of such TAA (Liang et al., 2013). Unleashing specific T-cell responses against OV-expressed TAA through combination with checkpoint inhibitors could prove to be a very valuable strategy.

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Other than JX-594, a large number of clinically active and pre-clinical oncolytic viruses have been tested in HCC models, yet precious few of these agents have progressed into HCC-directed clinical trials. As in other fields, OV laboratory science races well ahead of clinical practice, and in this respect, anti-HCC oncolytic virotherapy is no different. The potential exists for the medicines regulatory authorities to approve multiple efficacious OV in HCC clinical practice, paving the way for stratified therapy. In order to realise this potential and reap the rewards, we must first push these pre-clinical agents into the clinic.

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Table 1 – HCC-specific oncolytic viruses; mechanisms of targeting

Targeting principle	Example	Description	Issues	Reference
Liver specific viral promoter	Transthyretin-promoter driven adenovirus	Transthyretin is a thyroid hormone transport protein, secreted into serum by hepatocytes.	Requires additional cancer specificity	(Hsieh et al., 2009)
HCC specific viral promoter	AFP-promoter-driven adenovirus	AFP is produced in high levels from the foetal liver and yolk sac, but not normally in adults.	AFP is frequently only expressed in a sparse population of HCC cells, and can also be expressed from non-malignant hepatocytes in chronic hepatitis and cirrhosis (Ohguchi et al., 1998) (Johnson, 2001).	(Zhang et al., 2012)
Enzyme-activated viral protein	MMP-activated MVF protein	MMP substrate site is inserted into MVF.	Efficacy dependent on tumour MMP expression. Could have broader cancer specificity	(Muhlebach et al., 2010)
miRNA mediated control of virus gene expression in normal liver cells	mir-122 regulated adenovirus	mir-122 binding sites inserted into the 3'untranslated region of an adenovirus type 5 E1A-luciferase transcription cassette.	mir-122 expression is preserved in HCV-induced HCC, potentially rendering mir-122 regulated adenovirus ineffective in this subset of patients (Varnholt et al., 2008).	(Cawood et al., 2009)

AFP (alpha fetoprotein); MMP (membrane metalloproteinase); MVF (Measles virus fusion); miRNA (micro-RNA)

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Figure 1 – Therapeutic gene products expressed by engineered replication-competent adenoviruses and tested in pre-clinical models of HCC. Infection of a malignant hepatocyte (illustrated by the large blue rectangular cell) by replication competent adenovirus results in the expression of engineered therapeutic genes. Suppressor of cytokine signalling (SOCS)-1 and SOCS3 inhibit JAK phosphorylation of STAT, thus attenuating cytokine signal transduction and suppressing tumour growth (Wei et al., 2011) (Liu et al., 2013). Tumour suppressor in lung cancer 1 (TSLC1) is a cell adhesion molecule whose overexpression inhibits cell growth and migration, and induces apoptosis (He et al., 2012). Numerous engineered genes enhance apoptosis: Melanoma differentiation associated gene (mda)-7/IL-24 binding to its receptor triggers mitochondrial dysfunction and apoptosis, whilst receptor-independent tumour suppression is achieved via the induction of sustained ER stress (Xiao et al., 2010).The hepatocellular carcinoma suppressor 1 (HCCS1) gene product activates the mitochondrial apoptotic pathway by inducing lysosomal protease efflux (Gan et al., 2008) (Zhang et al., 2008). SMAC (second mitochondria-derived activator of caspase) inhibits the activity of XIAP, a potent inhibitor of caspase activation that prevents apoptosis (Pan et al., 2007) (Pei et al., 2004). XIAP protein translation can also be knocked down using targeted short hairpin RNA (shRNA), sensitizing cells to pro-apoptotic signals such as tumour necrosis factor-related apoptosis inducing ligand (TRAIL) (Ye et al., 2005) (Pan et al., 2008). Other OV-encoded therapeutic proteins that act directly on malignant cells are the pro-apoptotic apoptin and the sodium-iodide symporter (NIS), a transmembrane glycoprotein, which transports out two sodium cations in return for one iodide anion. NIS proteins allow the intracellular concentration of radioactive iodide, inducing apoptosis (Zhang et al., 2012) (Grunwald et al., 2013). Several OV-encoded therapeutic proteins are secreted for paracrine effects on other cells within the tumour microenvironment; IL-12 drives the activation/differentiation of NK and T-cells, whilst the C-C chemokine ligand 5 (CCL5) induces NK cell activation and T-cell chemotaxis (Yang et al., 2012) (Li et al., 2013). Secreted endostatin acts on endothelial cells to inhibit migration and proliferation, and to induce apoptosis (Li et al., 2005a).

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Table 2 – Engineering OV to enhance delivery and survival in pre-clinical HCC models

Mechanism	Description	Potential advantages	Reference
Viral surface modification using polymers	Arginine-grafted bioreducible polymer or high molecular weight polyethylene glycol chemically conjugated to oncolytic adenovirus surface	Reduced hepatocyte infection and liver toxicity Reduced neutralisation by antibodies	(Kim et al., 2011) (Doronin et al., 2009)
Virus-mediated inhibition of NK and NKT cells	VSV expressing a protein from human cytomegalovirus known to downregulate CD155.	Reduced NK and NKT cell recruitment to the site of viral infection, reducing virus inactivation	(Altomonte et al., 2009)
Virus-mediated expression of chemokine-binding proteins	Recombinant VSV expressing high affinity chemokine-binding proteins; M3, from murine gammaherpesvirus-68, or equine herpes virus-1 glycoprotein G	Reduced neutrophil, NK and NKT cell recruitment to the site of viral infection, enhancing virus titres.	(Wu et al., 2008) (Altomonte et al., 2008)

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Table 3 – OV Tested in Pre-clinical Models of HCC

Virus species	Name	Modifications	Assessed in Clinical Trials?	Pre-clinical HCC Model	Reference
Parvovirus H-1	H-1PV	Wild-type	Yes, glioma	Cell lines	(Moehler et al., 2001)
HSV-1	G207	Deletion of both ICP34.5 neurovirulence genes & inactivation of ICP6 (ribonucleotide reductase) by insertion of the E.coli lacZ gene.	Yes; glioma	Cell lines and subcutaneous murine xenografts	(Song et al., 2006) (Xue et al., 2005)
	Cgal-Luc	Derived by repair of ICP4 (positive and negative regulation of virus genome) from CgalΔ3 virus, insertion of the LacZ gene into IGR54 and luciferase gene into IGR20.	No	Subcutaneous murine xenografts	(Argnani et al., 2011)
	H6-Luc	Derived from the H6 mutant; syncytium forming (Syn^-), benzhydrazone (glycosylation inhibitor) resistant. Luciferase cassette inserted into IGR20.	The closely related HF10 mutant has been tested in multiple solid tumours		
	G92A	ICP4 regulated by the albumin enhancer/promoter, mutated US3 gene (inhibitor of virus-induced apoptosis), disrupted thymidine kinase gene and insertion of the E.coli lacZ gene.	No	Orthotopic murine xenografts	(Chung et al., 2006)
	hrR3	ICP6 LacZ insertion mutant.	No		
Blue tongue virus	BTV-10	Wild-type, cell-culture adapted	No	Hep3B cell line	(Hu et al., 2008)
	BTV-HC_3	Wild-type, cell-culture adapted	No	Cell lines	(Chen et al., 2007)
Measles virus (Edmonston)	MV-CEA	Expresses extracellular domain of the human carcinoembryonic antigen (CEA)	Yes, glioma and ovarian cancer	Cell lines and subcutaneous murine xenografts	(Blechacz et al., 2006)
	MV-NIS	Expresses the human sodium iodide symporter (hNIS)	Yes, myeloma and multiple solid tumours		
	MV-GFP	Expresses green fluorescence protein. Human bone marrow-derived mesenchymal stem cells were infected with MV-GFP and systemically delivered in passively-immunised mice.	No	Orthotopic patient-derived HCC tissue xenografts	(Ong et al., 2013)
Newcastle Disease Virus	NDFLtag-EGFP	Derived from the wild-type LaSota vaccine strain. Carrying enhanced green fluorescence protein.	No	Human and murine hepatic stellate cells	(Li et al., 2009)
	NDV-Italien	Wild-type	No		
	rNDV/F3a a(L289A)	L289A mutation within the F (fusion) glycoprotein	No	Immunocompetent orthotopic murine model	(Altomonte et al., 2010)
	NDV/Anh-EGFP	Derived from the wild-type Anhinga strain. Carrying enhanced green fluorescence protein.	No	Cell lines and subcutaneous immunocompetent murine model	(Wu et al., 2014)
Vaccinia	GLV-1h68	Derived from the Lister strain and carries three gene cassettes: a Renilla luciferase-GFP (RUC-GFP) fusion cassette at the F14.5L locus, a reverse inserted human transferrin receptor and β-galactosidase cassette at the J2R locus (encodes thymidine kinase), and a β-glucuronidase cassette at the A56R locus (encoding hemagglutinin).	Yes, multiple solid tumours	Cell lines and murine xenografts	(Gentschev et al., 2011)
				Sorafenib-resistant cell lines	(Ady et al., 2014)
	JX963	Western reserve expressing GM-CSF, with double deleted thymidine kinase and vaccinia growth factor genes.	The closely related vvDD-CDSR expressing cytosine deaminase and somatostatin receptor is being tested in solid tumours	Orthotopic immunocompetent rabbit model	(Lee et al., 2009)

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Table 4 – Completed HCC-directed clinical trials using oncolytic viruses. Searches were performed on ClinicalTrials.gov, Current Controlled Trials, EU Clinical Trials Register and medline.

Virus	Phase	No of patients	Route	Delivered dose	Study Design	Anti-Cancer Effect	Grade III or IV Adverse Events	Reference
JX-594	2	25	IV followed by ITu	$1x10^9$ PFU	Single treatment group. IV day 1, ITu days 8 and 22, sorafenib day 25	mRECIST disease control rate 62% for JX-594 and 59% after initiation of sorafenib	Not available	(Heo et al., 2013b)
JX-594	2	30	ITu	$1x10^8$ or $1x10^9$ PFU	Randomised comparison between low and high dose JX-594	OS 14.1 months in high dose group Vs 6.7 months in low dose group (P=0.020)	Lymphopaenia, pyrexia hyperbilirubinaemia	(Heo et al., 2013a)
JX-594	2*	120	IV followed by ITu	$1x10^9$ PFU	JX-594 plus BSC or BSC only. IV day 1 followed by five ITu treatments	No significant overall survival advantage	Not available	(Transgene, 2013b)
Ad5 dl1520	2	10	IV followed by ITu	$3x10^{11}$ PFU	Randomised comparison between PEI and Ad5 dl1520	1 patient had PR by RECIST and 4 had PD	None	(Habib et al., 2002)

PEI (percutaneous ethanol injection); BSC (best supportive care); IV (intravenous); ITu (intratumoural); OS (overall survival); PFU (plaque forming units); PR (partial response); PD (progressive disease); RECIST(Response Evaluation Criteria in Solid Tumours); mRECIST (modified RECIST); * TRAVERSE trial

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Table 5 – Ongoing HCC-directed clinical trials using oncolytic viruses. Searches were performed on ClinicalTrials.gov, Current Controlled Trials and EU Clinical Trials Register.

Virus	Phase	No of patients	Route	Study Design	Primary objective(s)	Progress	Trial identifier
JX-594	2	21	IV	Single treatment group 5 x weekly infusions	Tumour response	Enrolment completed	NCT01636284
H101 recombinant human adenovirus type 5	3	120	HAI	Randomisation to adenovirus and TACE or TACE only	Overall survival	Recruiting	NCT01869088
VSV-hIFN-β	1	48	ITu	Modified "3+3" Fibonacci dose escalation	Maximum tolerated dose	Recruiting	NCT01628640

HAI (hepatic artery injection); TACE (trans-arterial chemo-embolization);

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Figure

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